Arthrobacter equi sp. nov., isolated from veterinary clinical material

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A Gram-positive-staining, catalase-positive, non-spore-forming, rod-shaped bacterium, strain IMMIB L-1606^T, isolated from genital swabs of a horse, was characterized using a polyphasic approach. Comparative 16S rRNA gene sequence analysis showed that the organism was related to members of the genus *Arthrobacter*, displaying sequence similarities of 93.5–99.1 % with the type strains of recognized species of the genus. Cell-wall analysis revealed peptidoglycan type A3 α L-Lys–L-Ser–L-Thr–L-Ala. DNA–DNA hybridization data and biochemical characterization of strain IMMIB L-1606^T enabled the isolate to be differentiated genotypically and phenotypically from phylogenetically closely related species of the genus *Arthrobacter*. Therefore, it is concluded that strain IMMIB L-1606^T represents a novel species of the genus *Arthrobacter*, for which the name *Arthrobacter equi* sp. nov. is proposed. The type strain of *Arthrobacter equi* sp. nov. is IMMIB L-1606^T (=DSM 23395^T=CCUG 59597^T).

The genus *Arthrobacter* consists of a group of Gram-positive, catalase-positive, aerobic, asporogenous bacteria. The main distinguishing morphological feature of species of the genus *Arthrobacter* is a growth cycle in which the irregular rods in young cultures are replaced by coccoid forms in older cultures (rod–coccus growth cycle). As presently circumscribed, the genus contains two groups of species referred to as the *Arthrobacter globiformis*/*Arthrobacter citreus* group (*Arthrobacter sensu stricto*) and the *Arthrobacter nicotianae* group (Jones & Keddie, 1992, 2006). These groups differ in their peptidoglycan structure and menaquinone composition (Stackebrandt *et al.*, 1983; Keddie *et al.*, 1986). The *A. globiformis*/*A. citreus* group contains the A3 α peptidoglycan variant and the *A. nicotianae* group possesses the A4 α peptidoglycan variant. At the time of writing, the genus *Arthrobacter* comprised 61 species with validly published names (Euzéby, 2010). The majority of these species have been isolated from various environmental sources. Several species have been isolated from human clinical specimens, such as *Arthrobacter albus* (Wauters *et al.*, 2000), *Arthrobacter creatinolyticus* (Hou *et al.*, 1998), *Arthrobacter cummingsii* (Funke *et al.*, 1996), *Arthrobacter luteolus* (Wauters *et al.*, 2000), *Arthrobacter*

sanguinis (Mages *et al.*, 2008), *Arthrobacter scleromae* (Huang *et al.*, 2005) and *Arthrobacter woluwensis* (Funke *et al.*, 1996; Bernasconi *et al.*, 2004). Two species, however, have been isolated from veterinary sources, namely *Arthrobacter gandavensis* (Storms *et al.*, 2003) and *Arthrobacter nasiphocae* (Collins *et al.*, 2002). During the course of study of bacterial isolates encountered in animal clinical specimens, an unusual strain, designated IMMIB L-1606^T, isolated from a horse, was characterized. In this paper, the results of a study of strain IMMIB L-1606^T using a polyphasic taxonomic approach are reported and a novel species of the genus *Arthrobacter* is proposed.

Strain IMMIB L-1606^T was isolated from genital swabs of a horse using Columbia agar (BD) supplemented with 5 % sheep blood. The strain was characterized taking into consideration the proposed minimal standards for the characterization of new members of the suborder *Micrococccineae* as suggested by Schumann *et al.* (2009). Motility was observed by the hanging drop technique for cells in exponential phase in brain-heart infusion (BHI) broth. Cell morphology was observed using a transmission electron microscope (Philips CM-10) with cultures grown on BHI broth medium at 35 °C for 17–190 h. Negative staining of cells was performed with 1 % uranyl acetate. Growth was tested on diagnostic sensitivity test agar (DST agar; Oxoid), BHI agar (BD), Columbia agar supplemented with 5 % sheep blood (BD) and tryptic soy agar (TSA;

The GenBank/EMBL/DDBJ accession number for the 16S rRNA gene sequence of strain IMMIB L-1606^T is FN673551.

Two supplementary figures are available with the online version of this paper.

Oxid). Growth at various temperatures (5, 10, 22, 30, 37 and 42 °C) was determined on Columbia agar. Growth in NaCl was determined in nutrient broth (Difco). The pH range and optimum were determined by incubation in BHI broth at pH 4.0, 5.0, 6.0, 7.4, 8.0 and 9.0. Oxidase activity was determined using the BBL DrySlide oxidase test (BD). The presence of catalase was assessed using 3 % (v/v) H₂O₂. The isolate and strains of phylogenetically related species (*Arthrobacter defluvii* DSM 18782^T, *Arthrobacter chlorophenolicus* DSM 12829^T, *Arthrobacter polychromogenes* DSM 20136^T and *Arthrobacter oxydans* DSM 20119^T) were characterized biochemically under the same laboratory conditions using the API Coryne and API ZYM systems according to the manufacturer's instructions. The Voges–Proskauer reaction was carried out using the method of Barritt (1936). Hippurate hydrolysis was tested using the method of Luechtefeld & Wang (1982). Hydrolysis of adenine, casein, elastin, guanine, hypoxanthine, testosterone, tyrosine and xanthine was investigated as described previously (Gordon, 1966, 1967). Aesculin hydrolysis was tested as described by Gordon (1966) and gelatin hydrolysis was tested as detailed by Gordon & Mihm (1957). DNase test agar (Difco) was used for the DNase assay. Starch hydrolysis was assessed by using the method of Barrow & Feltham (1993). Tests to determine carbon source utilization were performed as described previously (Yassin *et al.*, 1995).

Fatty acid methyl ester analysis was performed on the studied strains according to methods described previously (Minnikin *et al.*, 1980; Yassin *et al.*, 2007). The menaquinone composition of strain IMMIB L-1606^T was determined as described by Yassin & Hupfer (2006). Phospholipids were extracted, purified and identified using two-dimensional TLC as described previously by Yassin *et al.* (1993). The DNA G + C content (mol%) was determined by HPLC according to Mesbah *et al.* (1989). The peptidoglycan was isolated after disruption of cells by shaking with glass beads and subsequent trypsin digestion according to Schleifer (1985). Amino acids and peptides in cell-wall hydrolysates were analysed by two-dimensional ascending TLC on cellulose plates by using previously described solvent systems (Schleifer, 1985). Amino acid identity was confirmed and their molar ratio was estimated by GC (GC 14A; Shimadzu) and GC-MS (320-MS Quadrupole; Varian) of *N*-heptafluorobutyryl amino acid isobutyl esters (MacKenzie, 1987; Groth *et al.*, 1996).

The 16S rRNA gene of the isolate was amplified by PCR using previously described procedures (Rainey *et al.*, 1996) and sequenced directly using a *Taq* DyeDeoxy terminator cycle sequencing kit (Applied Biosystems) and an automatic DNA sequencer (model 310; Applied Biosystems). The closest known relatives of the novel isolate were determined by performing database searches. The 16S rRNA gene sequence of strain IMMIB L-1606^T and those of other known related bacteria, retrieved from GenBank, were added to the ARB database (Ludwig *et al.*, 2004) and aligned using the respective tool from the ARB package. The

resulting alignment was corrected manually and phylogenetic trees were constructed using maximum-parsimony (Fitch, 1971), neighbour-joining (Saitou & Nei, 1987) and maximum-likelihood (Felsenstein, 1981) methods. The evolutionary distance matrix for the neighbour-joining method was calculated using the correction of Jukes & Cantor (1969). Topologies of the neighbour-joining tree were evaluated using bootstrap analyses (Felsenstein, 1985) based on 1000 resamplings.

To investigate the phylogenetic relationships between strain IMMIB L-1606^T and members of other taxa, comparative 16S rRNA gene sequencing was performed. The almost-complete 16S rRNA gene sequence of the strain (1487 nt) was determined in this study. Sequence database searches (BLAST) indicated that strain IMMIB L-1606^T was most closely related to members of the genus *Arthrobacter* (sequence similarities of 93.5–99.1 %). A tree constructed using the neighbour-joining method depicting the phylogenetic position of strain IMMIB L-1606^T among members of the genus *Arthrobacter* is shown in Fig. 1 and shows that the isolate represents a distinct subline within the genus. Strain IMMIB L-1606^T clustered together with *A. defluvii* and *Arthrobacter niigatensis*, an association that was supported by a bootstrap resampling value of 74 % (Fig. 1). Highest 16S rRNA gene sequence similarities were shown with *A. defluvii* DSM 18782^T (99.1 %), *A. niigatensis* LC4^T (99.0 %), *A. chlorophenolicus* DSM 12829^T (98.8 %) and *Arthrobacter phenanthrenivorans* DSM 18606^T (98.3 %).

To investigate the genetic relationship between isolate IMMIB L-1606^T, *A. defluvii*, *A. niigatensis* and *A. chlorophenolicus*, chromosomal DNA–DNA hybridizations were performed. Reassociation values for strain IMMIB L-1606^T with respect to *A. defluvii* DSM 18782^T and *A. chlorophenolicus* DSM 12829^T were 32.3 % and 53.9 %, respectively. These results show that strain IMMIB L-1606^T belongs to a separate genomic species (Wayne *et al.*, 1987). DNA–DNA hybridization with *A. niigatensis* JCM 21826^T (Ding *et al.*, 2009) was not performed as this strain is not available since its authenticity has not been verified by the JCM or DSM culture collections.

Strain IMMIB L-1606^T was aerobic, catalase-positive and oxidase-negative. Colonies were cream coloured on Columbia agar supplemented with 5 % sheep blood, BHI agar, DST agar and TSA. Strain IMMIB L-1606^T exhibited a rod–coccus cycle. Cells were predominantly short rods or oval-shaped (0.56–0.72 × 2.0–2.3 µm) after 17–60 h growth. The rods fragmented into cocci (0.49–0.88 µm in diameter) after 190 h growth (Supplementary Fig. S2 in IJSEM Online). Cells were Gram-positive and grew between 10 and 35 °C. The Voges–Proskauer reaction was negative. The isolate hydrolysed adenine, casein, gelatin, hypoxanthine, tyrosine and urea, but did not reduce nitrate. Using the API Coryne system, the isolate did not produce acid from glucose or any other carbohydrates tested. Using the API Coryne and API ZYM systems, activity was detected for β-galactosidase, α-glucosidase, β-glucuronidase, leucine

Fig. 1. Neighbour-joining phylogenetic tree based on 16S rRNA gene sequences showing the position of strain IMMIB L-1606^T within the radiation of species of the genus *Arthrobacter*. Numbers at the nodes indicate the level of bootstrap support >70% based on neighbour-joining analyses of 1000 resampled datasets; solid circles indicate that the corresponding nodes (groupings) were also recovered in maximum-likelihood and maximum-parsimony trees. Bar, 1.0% sequence divergence.

arylamidase, naphthol-AS-BI-phosphohydrolase and pyrazinamidase. All other enzyme tests in the API Coryne and API ZYM systems were negative. The isolate differed from *A. defluvii* and *A. chlorophenolicus* in the hydrolysis of hippurate and hypoxanthine (Table 1).

Cell wall analysis revealed that peptidoglycan hydrolysates (4 M HCl, 100 °C, 16 h) contained lysine, alanine, threonine, serine and glutamic acid in an approximate molar ratio of 0.9:2.6:0.9:0.7:1.0. The peptides L-Ala-D-Glu, L-Ala-D-Ala, L-Ala-L-Thr, L-Lys-L-Ser, L-Lys-D-Ala and

Table 1. Differential biochemical characteristics of strain IMMIB L-1606^T and phylogenetically related species of the genus *Arthrobacter*

Strains: 1, IMMIB L-1606^T; 2, *A. defluvii* DSM 18782^T; 3, *A. chlorophenicus* DSM 12829^T; 4, *A. polychromogenes* DSM 20136^T; 5, *A. oxydans* DSM 20119^T. All strains were positive for: hydrolysis of adenine, casein, gelatin, tyrosine and urea; utilization of cellobiose, citrate, gluconate, *myo*-inositol, L-lactate, lactose, maltose, melezitose, L-rhamnose, trehalose and D-xylose as carbon sources; utilization of L-alanine, L-proline and L-serine as carbon and nitrogen sources; and β -galactosidase, α -glucosidase, leucine arylamidase, naphthol-AS-BI-phosphohydrolase and pyrazinamidase activities. All strains were negative for: hydrolysis of elastin, guanine, keratin and testosterone; utilization of adipic acid, adonitol, *meso*-erythritol, *m*-hydroxybenzoate and 1,2-propanediol as carbon sources; utilization of acetamide and ornithine as sources of carbon and nitrogen; esterase C4, ester lipase C8, lipase C14, α -glucosidase, β -glucosidase, β -galactosidase, *N*-acetyl- β -glucosaminidase, valine arylamidase, cystine arylamidase, trypsin, chymotrypsin, α -mannosidase, α -fucosidase and urease activities; and acetoin production. +, Positive; –, negative; w, weak utilization after 3 weeks. All data are from this study. Note that data on glucose utilization deviate from those originally published by Kim *et al.* (2008) due to the test methods.

Characteristic	1	2	3	4	5
Hydrolysis of:					
Aesculin	–	+	+	+	–
Hippurate	+	–	–	–	–
Hypoxanthine	+	–	–	–	–
Xanthine	–	+	–	–	–
DNA (2 days)	+	–	+	–	–
Starch (3 days)	+	+	–	+	–
Utilization as source of carbon and energy:					
Acetate	+	–	–	–	–
iso-Amyl alcohol	–	+	–	–	–
L-Arabinose	+	–	+	+	+
2,3-Butanediol	+	+	–	–	+
D-Galactose	+	–	+	+	+
D-Glucose	+	–	+	+	+
<i>p</i> -Hydroxybenzoate	w	+	–	–	+
D-Mannitol	+	–	+	+	+
Raffinose	+	w	+	+	+
Sucrose	+	–	+	+	–
D-Sorbitol	+	–	+	+	+
Utilization as source of carbon and nitrogen:					
Arginine	+	+	–	+	–
Production of:					
Acid phosphatase	–	+	–	–	–
Alkaline phosphatase	–	–	–	+	+
β -Glucuronidase	+	+	+	+	–
Gelatin liquefaction	+	+	+	+	+
Pyrrolidonyl arylamidase	–	+	–	–	–
Nitrate reduction	–	+	–	+	+

L-Ser–L-Lys–D-Ala were detected in the partial hydrolysate of the peptidoglycan (4 M HCl, 100 °C, 0.75 h). Dinitrophenylation according to Schleifer (1985) revealed that alanine was at the N terminus of the interpeptide bridge. From the available data, it can be concluded that the peptidoglycan of strain IMMIB L-1606^T is of type A3 α L-Lys–L-Ser–L-Thr–L-Ala (Schleifer & Kandler, 1972; A11.23 according to http://www.dsmz.de/microorganisms/main.php?content_id=35). The fatty acid profile of strain IMMIB L-1606^T was dominated by ai-C_{15:0} (47.5%), i-C_{15:0} (24.5%), i-C_{16:0} (8.5%) and ai-C_{17:0} (4.8%). These results are in accordance with the fatty acid compositions of other members of the genus *Arthrobacter*. Table 2 shows the concentrations of various cellular fatty acids in *A. defluvii*, *A. chlorophenicus*, *A. polychromogenes* and *A. oxydans*. The four organisms clearly differ from each other and from strain IMMIB L-1606^T in the proportions of the different fatty acids. The polar lipids of strain IMMIB L-1606^T included diphosphatidylglycerol, phosphatidylglycerol, phosphatidylinositol, two unknown phospholipids and two unknown glycolipids (Supplementary Fig. S1, available in IJSEM Online), i.e. phospholipid type PI according to Lechevalier *et al.* (1977). The menaquinone composition of strain IMMIB L-1606^T revealed that MK-9(H₂) was the predominant component. A substantial amount of MK-8(H₂) and traces of MK-7(H₂) were also detected. The DNA G+C content of strain IMMIB L-1606^T was 67.0 mol%, which is within the range (65.5–68.2 mol%) previously obtained for species of the genus *Arthrobacter*. The chemotaxonomic profile generated in the present study confirmed the affiliation of strain IMMIB L-1606^T with the genus *Arthrobacter*.

Genomic and chemotaxonomic data confirm that strain IMMIB L-1606^T belongs to the genus *Arthrobacter*. On the basis of low DNA–DNA relatedness with its closest phylogenetic neighbour and differences in biochemical and morphological traits with respect to all of the type strains described for the genus *Arthrobacter*, it can be concluded that strain IMMIB L-1606^T represents a novel species within the genus, for which the name *Arthrobacter equi* sp. nov. is proposed.

Description of *Arthrobacter equi* sp. nov.

Arthrobacter equi (e' qui. L. gen. n. *equi* of the horse).

Cells are Gram-positive-staining, non-motile, non-spore-forming and have a rod–coccus cycle. Cells are aerobic, catalase-positive and oxidase-negative. Colonies are cream-coloured, convex and smooth with entire margins, reaching 1–2 mm in diameter on Columbia blood agar supplemented with 5% sheep blood, BHI agar, DST agar and TSA after 2 days of incubation at 34 °C. No growth occurs at 37 °C. Tolerates 0–2% NaCl. Grows at pH 6.0–9.0, with optimum growth at pH 6.0; does not grow at pH 4.0–5.0. Nitrate reduction is negative. Acetoin is not produced. Hydrolyses adenine, casein, DNA (2 days of

Table 2. Cellular fatty acid composition (%) of strain IMMIB L-1606^T and the most closely related *Arthrobacter* species

Strains: 1, IMMIB L-1606^T; 2, *A. defluvii* DSM 18782^T; 3, *A. chlorophenolicus* DSM 12829^T; 4, *A. polychromogenes* DSM 20136^T; 5, *A. oxydans* DSM 20119^T. All data are from this study. C_{17:0} was not detected in any strain studied. —, Not detected.

Fatty acid	1	2	3	4	5
i-C _{14:0}	2.9	8.5	2.4	3.5	1.0
C _{14:0}	0.9	1.9	1.1	1.0	0.4
i-C _{15:0}	24.5	—	17.2	6.6	9.7
ai-C _{15:0}	47.5	15.8	49.7	56.2	46.2
C _{15:0}	0.3	0.6	0.7	—	—
i-C _{16:1ω7c}	1.2	5.9	0.4	1.8	1.3
i-C _{16:0}	8.5	17.0	10.4	6.6	5.7
C _{16:1ω7c}	0.6	0.2	0.4	1.8	1.6
C _{16:0}	2.3	9.5	4.4	3.1	4.5
i-C _{17:1}	0.8	0.7	—	—	0.6
ai-C _{17:1}	1.4	4.9	0.6	3.1	4.4
i-C _{17:0}	2.3	1.9	1.6	0.7	1.2
ai-C _{17:0}	4.8	23.5	8.5	7.8	17.5
i-C _{18:1}	—	—	—	—	0.2
C _{18:1ω9c}	0.2	0.5	—	—	2.0
C _{18:0}	0.1	0.9	0.2	—	0.5

incubation), gelatin, hippurate, hypoxanthine, starch (3 days of incubation), tyrosine and urea, but not aesculin, elastin, guanine, keratin, testosterone or xanthine. Acetate, L-arabinose, 2,3-butanediol, cellobiose, citrate, D-galactose, D-glucose, D-gluconate, *p*-hydroxybenzoate, *myo*-inositol, L-lactate, lactose, maltose, D-mannitol, melezitose, raffinose, L-rhamnose, D-sorbitol, sucrose, trehalose and D-xylose are utilized as carbon sources, but adipate, adonitol, isoamylalcohol, *meso*-erythritol, *m*-hydroxybenzoate and 1,2-propanediol are not utilized as carbon sources. L-Alanine, gelatin, L-proline and L-serine are utilized as simultaneous sources of carbon and nitrogen, but acetamide and ornithine are not utilized. Acid is not produced from D-glucose, glycogen, lactose, D-mannitol, maltose, D-ribose, sucrose or D-xylose. Activity is detected for β -galactosidase, α -glucosidase, β -glucuronidase, leucine arylamidase, pyrazinamidase and naphthol-AS-BI-phosphohydrolase. No activity is detected for alkaline phosphatase, acid phosphatase, α -chymotrypsin, esterase C4, ester lipase C8, α -galactosidase, β -glucosidase, *N*-acetyl- β -glucosaminidase, α -fucosidase, lipase C14, α -mannosidase, cystine arylamidase, pyrrolidonyl arylamidase, valine arylamidase, trypsin and urease. The cell-wall peptidoglycan type is A3 α (Schleifer & Kandler, 1972) with an L-Lys-L-Ser-L-Thr-L-Ala interpeptide bridge. The major menaquinone is MK-9(H₂). The polar lipids are diphosphatidylglycerol, phosphatidylglycerol, phosphatidyl-inositol, two unknown phospholipids and two unknown glycolipids. The major fatty acids are ai-C_{15:0} and i-C_{15:0}.

The type strain is IMMIB L-1606^T (=DSM 23395^T=CCUG 59597^T), isolated from a genital swab of a horse. The DNA G + C content of the type strain is 67.0 mol%.

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